

Effect of diabetes and aminoguanidine therapy on renal advanced glycation end-product binding

SHERIF YOUSSEF, DOMINIQUE T. NGUYEN, TINA SOULIS, SIANNA PANAGIOTOPOULOS, GEORGE JERUMS, and MARK E. COOPER

Department of Medicine, University of Melbourne, and the Endocrine Unit, Austin and Repatriation Medical Centre (Austin Campus), Heidelberg, Victoria, Australia

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Background. Advanced glycation end-products (AGEs) have been implicated in the pathogenesis of diabetic nephropathy, and aminoguanidine (AG) has been shown to decrease the accumulation of AGEs in the diabetic kidney.

Methods. This study investigates changes in AGE binding associated with diabetes in the rat kidney using *in vitro* and *in vivo* autoradiographic techniques. Male Sprague-Dawley rats were randomized into control and diabetic groups with and without AG treatment and were sacrificed after three weeks. Frozen kidney sections (20 μ m) were incubated with [¹²⁵I]-AGE-RNase or [¹²⁵I]-AGE-BSA. To localize the AGE binding site, *in vivo* autoradiography was performed by injection of 15 μ Ci of [¹²⁵I]-AGE-BSA into the abdominal aorta of the rat.

Results. Low-affinity binding sites specific for AGEs in the renal cortex ($IC_{50} = 0.28 \mu$ M) were detected by *in vitro* autoradiography. There was a significant increase in [¹²⁵I]-AGE binding in the diabetic kidney, which was prevented by AG treatment. Emulsion autoradiography revealed that binding was localized primarily to proximal tubules in the renal cortex. Renal AGE levels, as assessed by fluorescence or by radioimmunoassay, were increased after three weeks of diabetes. This increase was attenuated by AG therapy.

Conclusions. AGE binding sites are present within the proximal tubules of the kidney and appear to be modulated by endogenous AGE levels. It remains to be determined if these binding sites represent receptors involved in clearance of AGEs or are linked to pathogenic pathways that lead to the development of diabetic nephropathy.

Advanced glycation is the nonenzymatic and irreversible glycation of proteins and lipids [1]. The pathway for nonenzymatic glycation begins with the interaction of glucose and biological proteins and is most prevalent in proteins with a long half-life. Aminoguanidine (AG) has

been shown to inhibit advanced glycation end-product (AGE) formation and cross-linking in the streptozocin diabetic rat [2]. Aminoguanidine does not interfere with the formation of Amadori products such as glycated hemoglobin (Hb), but acts to inhibit subsequent rearrangements that are essential for cross-linking reactions [3]. Hemoglobin in the presence of glucose has been shown *in vivo* to form advanced glycated Hb [4]. Furthermore, AG has been shown to decrease AGE-Hb, consistent with the action of AG being distal to the formation of glycated Hb (HbA1c) [4]. Advanced glycation end-products have been implicated in a number of biological processes, particularly in the pathogenesis of diabetic complications and aging [5].

The function of AGE-specific binding proteins has not been fully elucidated, although it has been suggested they may act as receptors and may lead to alterations in cell function [6, 7]. A number of AGE-binding proteins have been identified. These include a 35 kDa receptor, known as RAGE, which is a member of the immunoglobulin superfamily [8, 9]. Another AGE-binding protein is a lactoferrin-like molecule that is noncovalently associated with RAGE. At least four other AGE-binding proteins have been identified, including OST-40, 80K-H, Galectin 3 [10], and the antibacterial protein lysozyme [11].

This study has evaluated AGE binding sites in the kidney, a site of diabetic complications that appears to involve AGE-dependent pathways [2, 12], using both *in vitro* and *in vivo* autoradiographic techniques. Furthermore, the effects of diabetes and of AG therapy, maneuvers associated with changes in endogenous AGE levels [13], on [¹²⁵I]-AGE binding were also assessed. To assess the relationship between AGE accumulation and [¹²⁵I]-AGE binding in the kidney, AGE levels were simultaneously measured in the kidney.

METHODS

Experimental plan

Experimental animal models were set up as previously described [14]. Eight-week-old, male Sprague-Dawley rats

Key words: proximal tubule, AGEs, autoradiography, diabetic nephropathy, glucose control, hemoglobin.

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were randomized into four groups: control, control with AG (2 g/liter in drinking water; Regis, Morton Grove, IL, USA), diabetic (induced by intravenous injection of streptozocin 50 mg/kg after an overnight fast), and diabetic with AG (1 g/liter in drinking water). Rats were sacrificed after three weeks. Prior to sacrifice, rats were weighed, and blood pressure was determined by tail cuff plethysmography [15]. Only rats with plasma glucose of more than 15 mmol/liter were considered diabetic. For *in vitro* autoradiography, kidneys were removed and snap frozen in liquid nitrogen, and 20 μ m sections were cut on a cryostat and mounted on slides coated with gelatin (0.5%). For *in vivo* autoradiography experiments, animals were infused with radiolabeled AGEs before sacrifice (discussed later here).

Preparation of advanced glycation end-products

Fraktion V, free fatty acid (FFA), bovine serum albumin (BSA; Boehringer-Mannheim, Mannheim, Germany), or ribonuclease A (RNase) (Sigma Pharmaceuticals, St. Louis, MO, USA) was glycated *in vitro* by incubation at 37°C for 120 days with 0.5 M glucose in 0.2 M sodium phosphate buffer. The solutions were sterilized by passage through a 0.2 mm millipore membrane filter into a vacuette (vacuum sealed sterile test tube). This filtering step results in the removal of lower molecular weight substances, including free glucose.

Iodination of advanced glycation end-products

Thirty micrograms of AGE-BSA or AGE-RNase were iodinated using iodogen (1,3,4,6-tetrachloro-3 α ,6 α -diphenyl-glycouril; Pierce Chemical Co., Rockford, IL, USA). The mixture was allowed to react for 10 minutes. The entire mixture was removed from the iodogen-coated tube and was then added to 200 ml of 0.2% phosphate-buffered gelatin (PBG) [0.2% gelatin into phosphate-buffered saline (PBS)]. The mixture, including the labeled protein, was separated in a previously prepared 10 ml Sephadex G50 column.

In vitro autoradiography

Frozen tissues were cut at 20 μ m on a cryostat at -20°C. Tissue sections were washed twice for 7.5 minutes in a preincubation buffer containing 10 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), 100 mM NaCl, 5 mM KCl, 5 mM MgCl₂, 1 mM ethyleneglycol-bis-(β -amino-ethyl ether) N, N'-tetra acetic acid (EGTA), 0.1% BSA, and 0.5 mg/ml bacitracin. Tissue sections were then incubated for three hours in incubation buffer, which was made of prewash buffer and [¹²⁵I]-AGE tracer. Final counts in the incubation buffer were approximately 350 to 400 cpm/ μ l, with the total counts used being approximately 3.0×10^6 cpm and a final concentration of approximately 2 pM of [¹²⁵I]-AGE. Tissue sections were washed first in preincubation buffer for four minutes, followed by two four-minute washes in postincubation

buffer made up of 10 mM HEPES, 100 mM NaCl, 5 mM KCl, 5 mM MgCl₂, 1 mM EGTA, and 0.5 mg/ml bacitracin. Sections were dried at room temperature and were then placed under x-ray film for two weeks. Competitive binding studies were performed with varying concentrations of unlabeled AGEs. Competition studies were also performed with nonglycated proteins. Nonspecific binding (NSB) was determined by the addition of 10 μ M unlabeled AGEs ($\times 200$ tracer concentration). Data were obtained from two sections of five different kidneys for each experimental point and are shown as mean \pm SEM. In a separate series of experiments, cryostat sections were incubated with a different radioligand, [¹²⁵I]-AGE-RNase, and competition studies were performed with unlabeled AGE-RNase, AGE-BSA, and native (nonglycated) BSA and RNase.

Emulsion autoradiography

To localize AGE-binding sites, emulsion autoradiography was performed. Following postincubation washing, sections were fixed at 4°C for 30 minutes in 10% neutral-buffered formalin (NBF). Sections were briefly dipped at 42°C in LM-1 liquid emulsion (Amersham, Bucks, UK) diluted 1:1 with distilled water, dried for one to two hours at 25°C in less than 80% humidity. The emulsions were then exposed for at least two weeks at 4°C before development. Sections were then stained with hematoxylin and eosin.

In vivo autoradiography

To define further cellular localization of [¹²⁵I]-AGE binding in the renal cortex, the tracer was infused *in vivo* in separate groups of rats. This approach has been used when characterizing other binding sites because it is associated with better preservation of morphology [16, 17]. Rats were anesthetized with pentobarbital sodium (Nembutal 50 mg/kg body wt), and the abdominal cavity was opened with a midline incision. For infusion of [¹²⁵I]-AGEs, a 22-gauge catheter was inserted into the abdominal aorta below the renal arteries. For total binding studies, rats were infused with approximately 15 μ Ci [¹²⁵I]-AGE-BSA in 1 ml of 0.1 M PBS at pH 7.4. After 20 minutes of circulation, the rats were perfused at systolic blood pressure (approximately 140 mm Hg) with PBS for one minute, followed by 2% glutaraldehyde for two minutes. After perfusion fixation, one kidney was removed and fixed in 10% neutral-buffered formalin overnight before being embedded in paraffin. Nonspecific binding (NSB) was performed in separate animals by infusion of excess (10^{-5} M) unlabeled AGE proteins three minutes prior to the infusion of the [¹²⁵I]-AGE tracer. The procedure was then followed as mentioned earlier here. The other kidney was frozen, and 20 μ m sections were cut on a cryostat, then dried, and placed under x-ray film.

Paraffin-embedded sections were cut at 4 μ m and were

dewaxed by incubation in histolene for four minutes and then in decreasing ethanol followed by double-distilled water (ddH₂O). The sections were dried at room temperature and then were briefly dipped into LM-1 liquid emulsion at 42°C, diluted 1:1 with distilled water, dried for one to two hours at 25°C at less than 80% humidity. Slides were incubated for approximately two weeks, were developed in Phenisol for two minutes, and were fixed in Hypam for four minutes. After development, the slides were counterstained with hematoxylin for two minutes and eosin for 30 seconds.

Lectin staining

To define if the renal tubular binding of [¹²⁵I]-AGEs was localized to distal or proximal tubules, kidney sections were stained with lectin from phaseolus vulgaris (PHA-E, red kidney bean) [18], which specifically stains proximal tubules [19]. Sections were dewaxed through to double-distilled water, were then quenched with methanol for 20 minutes, and were washed three times with PBS. Endogenous sites were blocked by incubation with normal horse serum for 10 minutes. Tissue sections were then exposed to lectin (1 µg/ml) for 60 minutes. Secondary avidin-biotin complex (ABC) staining was then used with 3,3'-diaminobenzidine tetrahydrochloride (DAB) to localize lectin binding.

Measurement of advanced glycation end-products

As previously described [2], in parallel experiments using the same protocol, the cortex of the left kidney was removed, minced, and suspended in PBS, followed by centrifugation at 15,000 revolutions/min for 30 minutes at 4°C. Lipid extraction was performed by the addition of 5 ml of chloroform/methanol (2:1 vol:vol), followed by gentle shaking and overnight incubation at 4°C. The upper layer was removed, and the pellet was washed three times with methanol and distilled water. The pellet was then resuspended in 0.05 M acetic acid and 1 mg/ml pepsin, incubated for 18 hours at 4°C to remove noncollagenous proteins, and washed with 0.1 M CaCl₂, 0.02 M Tris-HCl (pH 7.6), and 0.05% toluene. Following digestion with type IV collagenase (0.1 mg/ml; Sigma) and proteinase K (0.1 mg/ml; Sigma) for 72 hours at 37°C, the samples were centrifuged at 15,000 revolutions/min for 30 minutes at 4°C. The resulting supernatant was then used to measure AGE levels.

Advanced glycation end-product levels in collagenase extracted renal homogenates were measured by specific fluorescence (370 nm/440 nm ex/em) as previously described [2] and by a radioimmunoassay (RIA) developed in our laboratory using an antibody directed against AGE-BSA [20]. Recent studies have revealed that this antibody detects the nonfluorescent AGE, carboxy methyl lysine-containing proteins [20]. AGE-RNase was used for preparation of tracer and standards. Collagenase-extracted re-

Table 1. Characteristics of animal models

Group	N	Body weight g	Blood pressure mm Hg	Blood glucose mmol/liter
Control	20	360 ± 16	114 ± 4	6.6 ± 0.2
Control + AG	8	334 ± 14	116 ± 3	5.4 ± 0.2
Diabetic	19	239 ± 11 ^b	129 ± 3 ^a	22.9 ± 1.2 ^b
Diabetic + AG	16	231 ± 17 ^b	134 ± 4 ^a	25.1 ± 1.7 ^b

Abbreviation AG is aminoguanidine. Data are shown as mean ± SEM.

^a *P* < 0.05, ^b *P* < 0.01 vs. Control

nal cortical homogenates were incubated for five hours at room temperature with the AGE-BSA antibody and tracer at room temperature. Sheep antirabbit IgG was then added and incubated overnight at room temperature. Precipitation of proteins was carried out by an addition of 8% polyethylene glycol. The minimum detectable concentration of AGE-RNase was 250 ng/ml, and the closing volume (CV) for this assay was 12.3% (*N* = 21) at a concentration of 2000 ng AGE-RNase/mg protein. Radioactivity was measured using a gamma counter (Cannberra Packard Instruments, St. Louis, MO, USA), and the amount of AGEs present was expressed as ng AGE-RNase. To determine the epitope reacting with our antibody, competition studies were performed as previously described [20]. These experiments indicated that the antibody detects advanced glycosylated RNase, advanced glycosylated BSA, and carboxy methyl lysine (CML)-containing proteins, including CML-BSA and CML-collagen. However, the antibody does not detect native BSA, native RNase, or pentosidine.

Analysis of results

All data are shown as mean ± SEM. The density of tracer binding was quantitated and analyzed by a computer-aided image analysis system (Imaging Research, St. Catherine's, Ontario, Canada). Because of the focal nature of [¹²⁵I]-AGE binding in the renal cortex, the mean of 20 to 25 quantitations was used for each kidney section. The *B*_{max}, which represents binding site density, and dissociation constant (*K*_d), which reflects the affinity of the ligand to the receptor, were assessed by the computer program LIGAND [21]. The program SIGMA-PLOT was used to determine the value for the half maximal inhibitory concentration (*IC*₅₀). Data were analyzed by analysis of variance on a Macintosh personal computer (Apple, Cupertino, CA, USA) using the StatView® program (Abacus Concepts Inc., Berkeley, CA, USA). A *P* value of less than 0.05 was considered to be statistically significant.

RESULTS

Characteristics of animals

Diabetes was associated with a reduced weight gain and a marked increase in plasma glucose levels (Table 1).

Blood pressure was modestly elevated in the diabetic rats. AG did not influence body weight, plasma glucose, or blood pressure.

[¹²⁵I]-AGE binding in the kidney

Maximal binding of [¹²⁵I]-AGEs was detected after two hours at a concentration of approximately 2 pM (specific activity of 100 Ci/mmol). In both control and diabetic rats, [¹²⁵I]-AGE binding was observed in the renal cortex, medulla, and papilla (Fig. 1). There was also prominent [¹²⁵I]-AGE binding in the kidneys at the corticomedullary junction. [¹²⁵I]-AGE binding was inhibited in the presence of 1×10^{-5} M unlabeled AGE [that is, nonspecific binding (NSB)], with NSB being less than 12% of maximal binding (Fig. 1E). NSB was assessed using different nonradiolabeled forms of AGEs, including AGE-BSA and AGE-RNase.

Using [¹²⁵I]-AGE-BSA as the radioligand, AGE-BSA competed for the tracer with a K_d of 1.95×10^{-7} M in the renal cortex (Fig. 2). AGE-RNase competed for the tracer with a K_d of 2.03×10^{-7} M. In contrast, native BSA and RNase did not displace the radioligand.

Using [¹²⁵I]-AGE-RNase as the radioligand, unlabeled AGE-RNase and AGE-BSA competed for the tracer. Nonglycated BSA or RNase failed to compete with [¹²⁵I]-AGE-RNase. The distribution of this tracer within the kidney and binding characteristics as obtained from analysis of the binding isotherms of the radioligand were similar to those observed with [¹²⁵I]-AGE-BSA (data not shown).

Diabetes was associated with an increase in [¹²⁵I]-AGE binding after three weeks, particularly in the renal cortex (Figs. 1C and 3A). However, there was no difference in the distribution of binding in the renal cortex, medulla, or papilla between diabetic and control rats. Aminoguanidine treatment did not influence [¹²⁵I]-AGE binding in control rats but prevented the increase in [¹²⁵I]-AGE binding in diabetic rats (Fig. 3). A similar pattern of binding was observed in the renal medulla (Figs. 1D and 3B), with an increase in [¹²⁵I]-AGE binding with diabetes and a prevention of this increase by AG treatment (Fig. 3B).

Analysis by the computer program LIGAND© revealed that there was an increase in the B_{max} in diabetes (Fig. 3) but no change in the dissociation constant (K_d , 2.03×10^{-7} M). Aminoguanidine decreased B_{max} but did not change K_d .

By *in vitro* emulsion autoradiography, it was confirmed that the major renal cortical binding site was to tubules in all groups studied, (Fig. 4). The binding appeared to be to proximal tubules. Therefore, to further localize [¹²⁵I]-AGE binding in the cortex more accurately, *in vivo* autoradiography was performed, supplemented by counterstaining with lectin from *Vaseolus vulgaris*, which specifically stains proximal tubules. *In vivo* binding revealed an almost threefold increase in renal cortex [¹²⁵I]-AGE binding in diabetes (control, 565 ± 50 ; diabetic, 1448 ± 56 dpm/mm², $P < 0.01$ vs. control). Furthermore, using emulsion autoradiography, the major site of binding was

in proximal tubules (Fig. 5 A, B), as confirmed by colocalization of grains representing [¹²⁵I]-AGE binding and lectin (Fig. 5C).

To further explore the relationship between [¹²⁵I]-AGE binding and AGEs, renal cortical AGE levels were measured both by fluorescence and RIA. Within three weeks of experimental diabetes, there was a twofold increase in AGEs as measured by either fluorescence (Fig. 6A) or RIA (Fig. 6B). This increase in AGEs in the diabetic rats was prevented by AG treatment. Aminoguanidine did not significantly influence AGE levels in control rats.

DISCUSSION

This study has identified a binding site for AGEs in the proximal tubules of the rat kidney, which appears to be specific for AGEs. Both radiolabeled AGE-BSA and AGE-RNase bound to this site. Furthermore, unlabeled AGE-BSA competed for the radioligand [¹²⁵I]-AGE-RNase, and AGE-RNase competed for the radioligand [¹²⁵I]-AGE-BSA. Importantly, native BSA or RNase did not displace either radioligand. Because both radioligands had a similar distribution within the kidney and similar binding characteristics, it is likely that they bound to the same binding site.

The number of proximal tubular AGE binding sites identified in this study was increased in experimental diabetes. Furthermore, this increase in AGE binding was prevented by AG treatment in the diabetic rats. Although there was an increase in maximal binding (B_{max}) in the diabetic kidney, there was no significant change in binding affinity. In this study, using both fluorescence and an RIA, which detects CML-containing proteins, it could be clearly demonstrated that within three weeks of induction of experimental diabetes that there is an increase in AGE levels in the kidney that did not occur with AG treatment. The concurrent increase in AGE levels and AGE binding is consistent with the hypothesis that endogenous AGEs are able to induce AGE binding sites in the kidney in experimental diabetes. Indeed, in blood vessels, it has been shown that increased expression of AGE proteins, as seen in diabetes, is associated with increased levels of the AGE receptor RAGE [22].

Gugliucci and Bendayan [23] have previously shown that AGE-containing proteins injected into rats are reabsorbed by proximal tubules. Therefore, it could be considered that AGE binding observed after *in vivo* injection represents nonspecific uptake by proximal tubular cells. However, a similar pattern of distribution of the radioligand was observed in the *in vitro* autoradiographic studies, and included repeated washes in buffer to displace endogenous ligands and proximal tubular enzymes involved in nonspecific protein reabsorption by renal proximal tubular cells. In the *in vivo* as well as the *in vitro* studies, excess unlabeled AGEs were associated with greater than 80% displacement of the radioligand, a phenomenon not observed with the native protein.

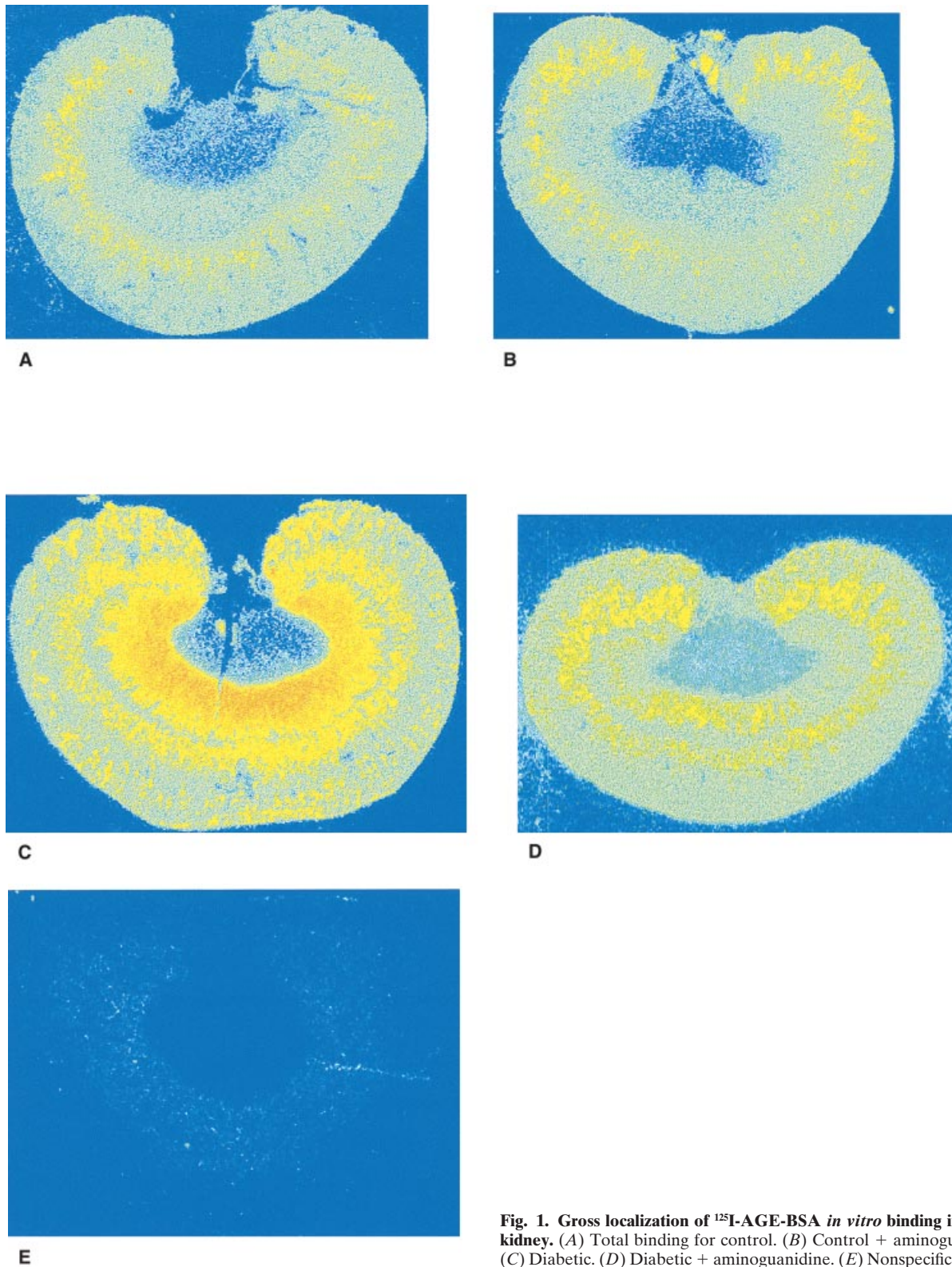


Fig. 1. Gross localization of ^{125}I -AGE-BSA *in vitro* binding in the rat kidney. (A) Total binding for control. (B) Control + aminoguanidine. (C) Diabetic. (D) Diabetic + aminoguanidine. (E) Nonspecific binding.

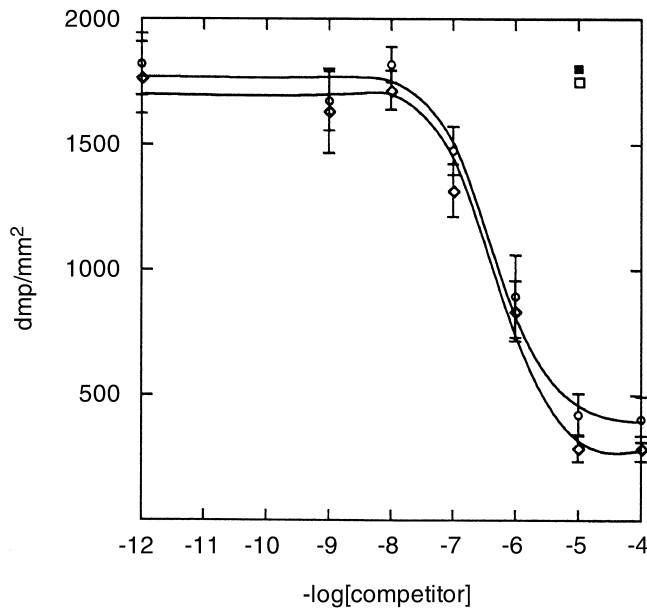


Fig. 2. Binding isotherms generated by *in vitro* incubation of tissue sections with ^{125}I -AGE-BSA with increasing concentrations of unlabeled AGE-BSA (\diamond), unlabeled AGE-RNase (\circ), native BSA (\square), and native RNase (\blacksquare). The y-axis represents binding density as quantitated by the MCID program.

Although this study identified a renal proximal tubular binding site in the kidney, it is difficult to determine if this site represents one of the previously characterized receptors or binding proteins for AGEs. Our own group has localized the receptor for AGEs known as RAGE in the kidney using immunohistochemical techniques [24]. It is unlikely that the binding site characterized in the current study is RAGE, because RAGE is localized primarily to distal tubules and to a lesser extent glomeruli rather than to proximal tubules.

In this study, diabetes was associated with increased AGE binding. A preliminary report has suggested that RAGE mRNA levels may be increased in experimental diabetes mellitus [25]. However, a recent report has suggested that there is no up-regulation of RAGE mRNA levels in the db/db diabetic mouse [26]. Li et al and Yang et al have identified three different AGE binding sites known as AGE-R1, AGE-R2, and AGE-R3 [10, 27], and AGE-R2 and AGE-R3 have been reported to be increased in mesangial cells from the NOD mouse model of diabetes [28]. There are no reports of these receptors being present in proximal tubular cells. Over the last few years, several other AGE-binding proteins have been identified, including lactoferrin and lysozyme [11, 29]. Of particular interest is the possibility that the proximal tubular binding may represent AGEs binding to lysozyme. Further studies are warranted to explore the possibility that the AGE-binding site identified in this study may represent one of the receptors described earlier here.

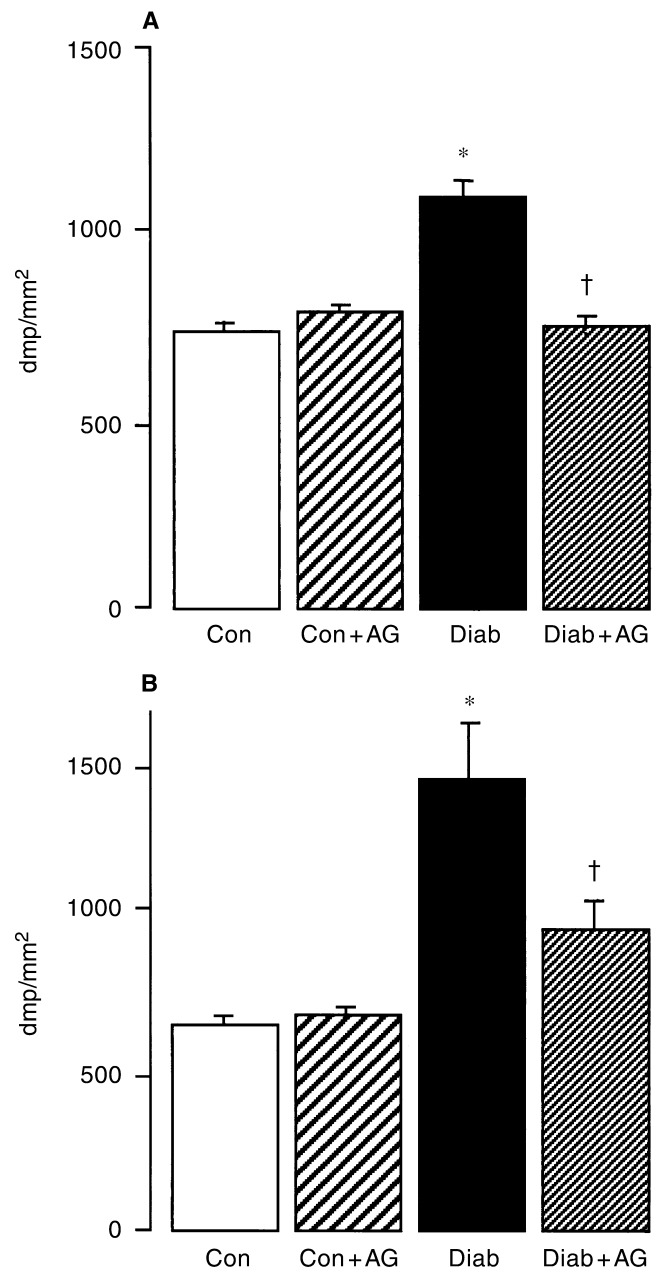


Fig. 3. *In vitro* ^{125}I -AGE-BSA binding in the renal cortex (A) and medulla (B). Data are shown as mean \pm SEM. Abbreviations are: Con, control; diab, diabetic; AG, aminoguanidine. * $P < 0.01$ vs. control, † $P < 0.01$ vs. diabetic.

The assessment of the binding isotherms revealed that the AGE binding site was of low affinity, with a K_d in the nanomolar range. This is consistent with the binding characteristics that have been described for RAGE, as well as for several of the other cloned AGE binding sites [9, 27, 30]. However, one must be cautious in comparing the findings from *in vitro* autoradiographic studies to those using membrane-binding techniques. For example, the assessment of binding sites in the brain for the pan-

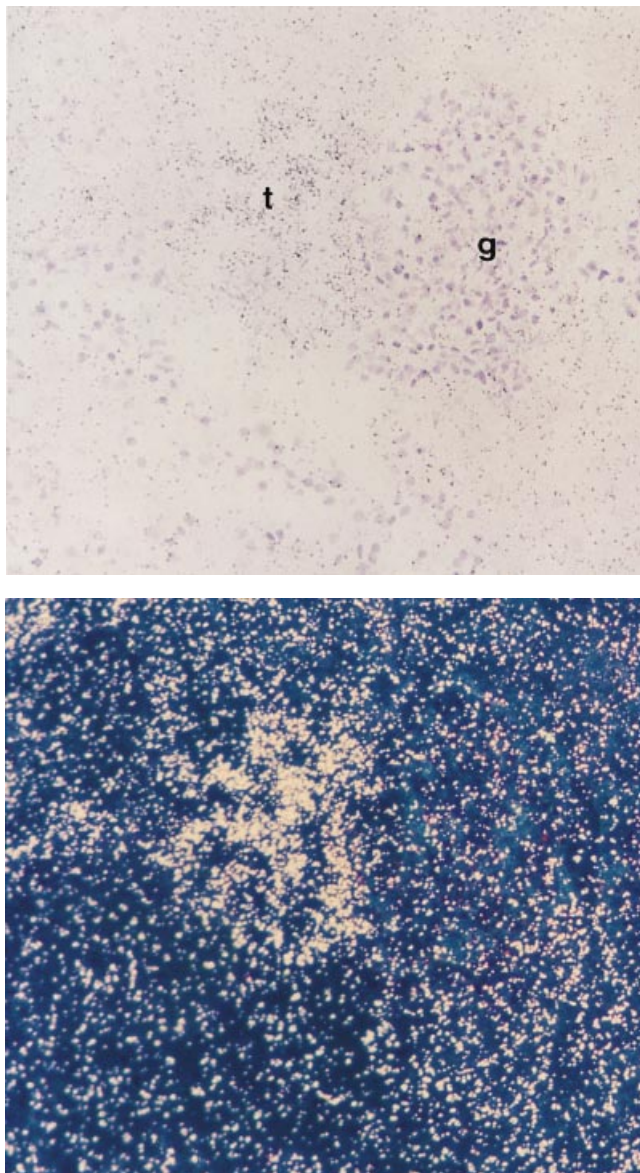


Fig. 4. *In vitro* emulsion autoradiography that shows binding to renal tubules (t) but not to glomeruli (g). (A) Bright field (magnification $\times 200$). (B) Dark field (magnification $\times 200$).

creatic hormone, amylin, revealed significant differences in the calculated affinity between membrane binding and *in vitro* autoradiographic studies [31].

In this study, AGEs were already elevated within three weeks of experimental diabetes mellitus. A similar early rise in AGE levels was reported by our group in vessels from diabetic rats using the same RIA as well as by immunohistochemistry [14, 24]. This suggests that AGEs that are either fluorescent or CML-containing appear early after the induction of diabetes mellitus. Hill et al have reported accumulation of AGEs in skeletal muscle arterioles within four to six weeks of experimental diabetes [32]. Furthermore, it has been shown that the effects

of AGEs are manifest early in experimental diabetes mellitus [32, 33]. There was a tendency for the increase in AGEs in the diabetic kidney to be greater, as assessed by RIA when compared with fluorescence measurements (Fig. 6). It should be noted that the RIA used in this study also detects CML [20], a nonfluorescent AGE [34]. Therefore, it is possible that CML-containing AGEs are increased earlier than fluorescent, cross-linked AGEs in response to chronic hyperglycemia in experimental diabetes. Further studies are required to characterize the chemical structure of the various AGEs formed *in vivo* that are detected by the different assay methods.

It is difficult to determine if the AGE binding site described in this study represents a clearance mechanism or a pathway leading to tissue injury. Gugliucci and Bendayan have suggested that the localization of AGE peptides in the proximal tubular cells is increased in diabetes and may lead to tubulointerstitial fibrosis [23]. More recently, it has been shown that one of the AGEs that circulates is localized to proximal tubules of rat kidney [35]. These findings provide further evidence that AGEs, including pentosidine, are reabsorbed by the proximal tubules of the kidney, degraded or modified, and finally excreted in the urine.

It has been demonstrated that AGEs can activate a range of intracellular second messengers, including mitogen-activated protein kinase in a renal tubular cell line [36]. This is consistent with the possibility that tubular cells respond to AGEs and result in a cascade of intracellular events, including expression of cytokines such as the prosclerotic growth factor transforming growth factor- β (TGF- β). Indeed, TGF- β has been shown to be induced by exogenous AGEs [37] and in endogenous states of increased AGEs such as experimental diabetes mellitus [14]. Tubulointerstitial fibrosis is a well-known pathological feature occurring in association with glomerulosclerosis in diabetic nephropathy [38].

It has been previously shown that several of the AGE-binding proteins act as receptors and lead to a range of intracellular processes, including activation of cytokines, that could cause tissue injury [39–42]. Our group has previously shown that AG can retard the development of experimental diabetic nephropathy [2]. In this study, AG treatment was associated with reduced AGE binding in the kidney. If one assumes that this renal proximal tubular binding site is involved in mediating tissue injury, one could speculate that the renoprotective effects of AG may involve reduced expression of receptors, which are mediating pathways relevant to the genesis of diabetic complications. The role of AG in mediating renoprotection in diabetes remains under clinical investigation [12]. Furthermore, a range of new inhibitors of advanced glycation is now under development, including novel inhibitors of AGE formation [43] and cross-link breakers that cleave preformed cross-links [44].

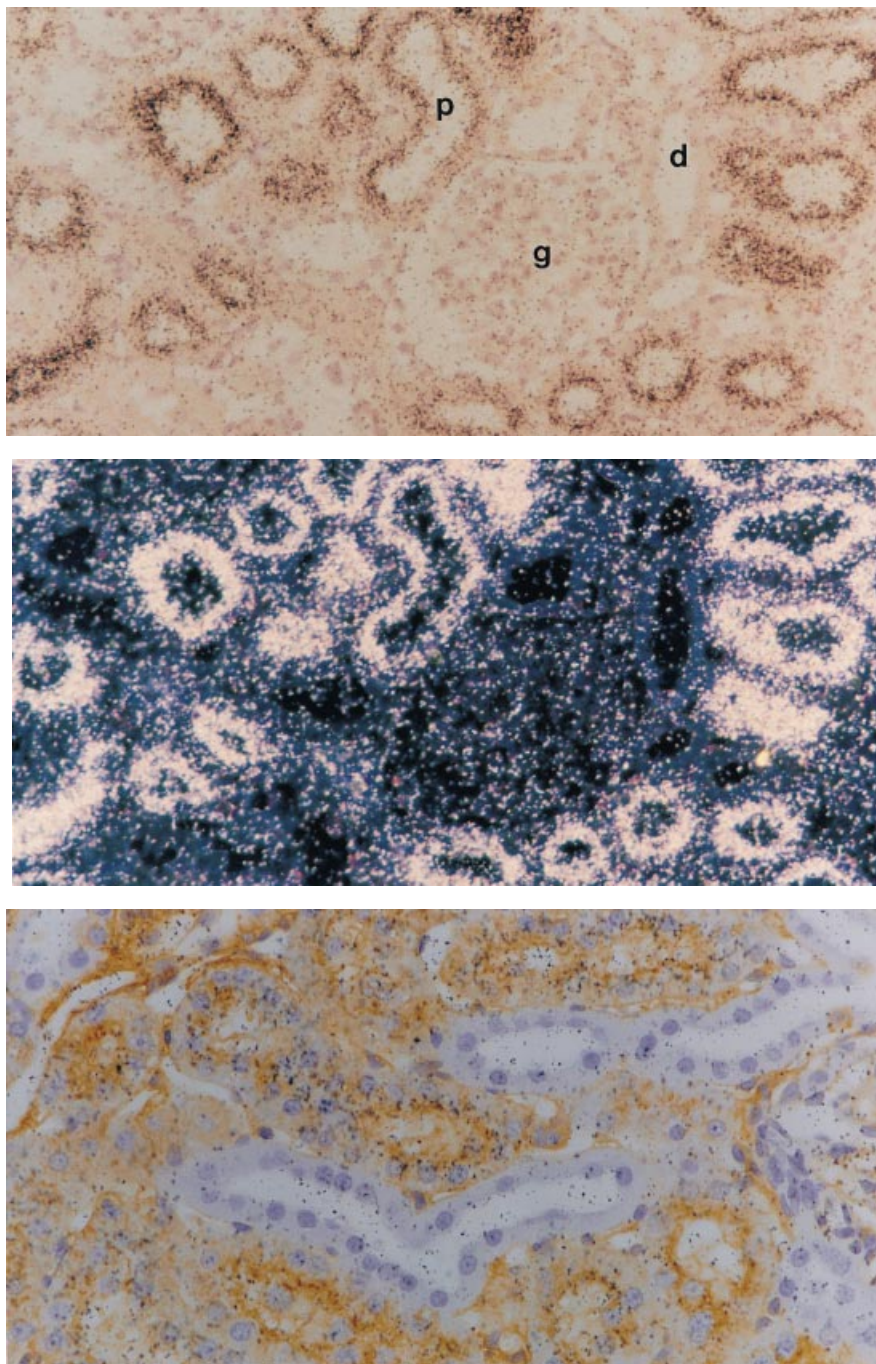


Fig. 5. *In vivo* emulsion autoradiography that shows binding to proximal tubules (p) but not to glomeruli (g) or distal tubules (d). (A) Bright field (magnification $\times 200$). (B) Dark field (magnification $\times 200$). (C) Bright field with lectin counterstain (magnification $\times 400$).

It cannot be excluded that the increase in AGE binding in diabetes represents a response to the increase in circulating and local AGE levels observed with chronic hyperglycemia. One could speculate that the increase in AGE binding represents a protective mechanism, activated in response to AGE formation, that operates in the diabetic kidney to clear potentially pathogenetic AGE proteins. Therefore, it remains to be determined if the increase in AGE binding indicates a pathway involved

in AGE clearance or in mediating potentially damaging effects on the kidney relevant to the genesis of diabetic nephropathy.

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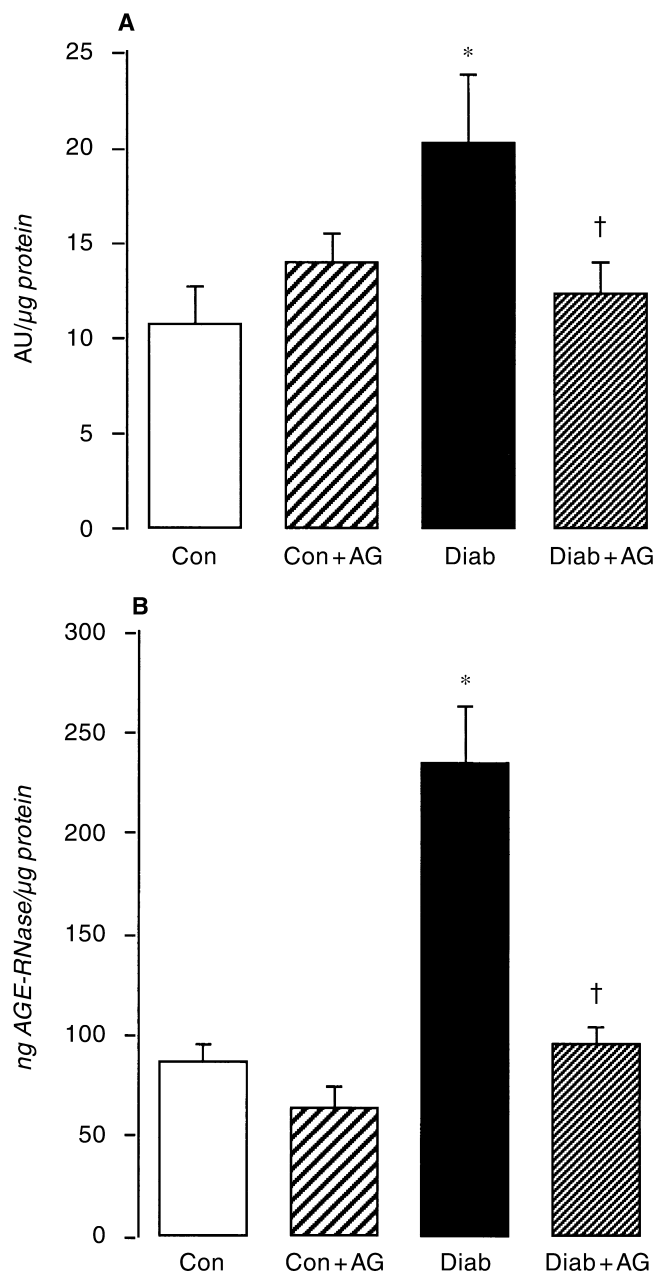


Fig. 6. AGE levels in renal cortical homogenates as measured by fluorescence (A) and radioimmunoassay (B). Abbreviations are: Con, control; diab, diabetic; AG, aminoguanidine. * $P < 0.01$ vs. control, † $P < 0.01$ vs. diabetic.

Reprint requests to Dr. Mark E. Cooper, Department of Medicine, Austin and Repatriation Medical Center, Repatriation Campus, West Heidelberg, Vic, 3081, Australia.
E-mail: cooper@austin.unimelb.edu.au

APPENDIX

Abbreviations used in this article are: ABC, avidin-biotin complex; AG, aminoguanidine; AGEs, advanced glycation end-products; B_{max} , binding site density; BSA, bovine serum albumin; CML, carboxy methyl lysine; DAB, 3,3'-diaminobenzidine tetrahydrochloride; ddH₂O, double

distilled water; FFA, free fatty acid; Hb, hemoglobin; IC₅₀, half maximal inhibitory concentration; K_d , dissociation constant; LF-L, lactoferrin-like; NBF, neutral buffered formalin; NSB, nonspecific binding; PBG, phosphate buffered gelatin; PBS, phosphate buffered saline; RAGE, AGE binding protein 35 kD receptor; RIA, radioimmunoassay; RNase, ribonuclease A; SD, Sprague-Dawley; TGF- β , transforming growth factor- β .

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